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Pathogenesis of a phleboviral infection (Punta Toro virus) in golden Syrian hamsters

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Summary. The hamster, *Mesocricetus auratus*, was examined as a possible model for investigating the poorly defined pathogenesis of the family *Bunyaviridae*, genus *Phlebovirus*. Punta Toro virus (PTV) isolates from Eastern Panama were highly virulent for two outbred and five inbred hamster strains, while isolates from western Panama were of low virulence. The Adames strain (eastern Panama) of PTV (LD_{50} approximately 1 PFU, sc) caused an acute fatal disease (average survival time, 3.8 days) in 10-week-old Lak:LVG(SYR) hamsters. Severe necrosis of the liver, spleen, and small intestine was associated with extensive expression of viral antigen in these organs. The Balliet strain (western Panama) of PTV ($LD_{50} > 6 \log_{10}$ PFU, subcutaneously) caused a mild hepatocellular infection with peak viral liver titers of 3–4 $\log_{10}$ PFU/g compared to 8–9 $\log_{10}$ PFU/g for the Adames strain. We observed histological lesions in the red pulp of the spleen or the lamina propria of the small intestine with the Adames strain. Lesions in the hamsters had characteristics of disseminated intravascular coagulation (DIC). The PTV-hamster model shares similarities to Rift Valley fever (phleboviral disease), which causes fatal disease in man and domesticated ruminants.

Introduction

Punta Toro virus (PTV) is one of eight members of the family *Bunyaviridae*, genus *Phlebovirus*, which produce disease in man [13, 6]. With the exception of Rift Valley fever virus (RVFV), which can be fatal for man as well as domesticated ungulates [14], most cause acute, febrile, nonfatal illness. A Punta Toro-mouse model has been described [15], but is highly age-dependent; lacks some of the histological lesions seen with RVFV infections; and because of the

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age-dependency, has limited use in protection studies after immunization. As a member of the family *Bunyaviridae*, PTV has a tripartite genome and reassortants can be made in the laboratory with PTV [5]. An adult model that demonstrates a large difference in PTV strain pathogenicity would be helpful in elucidating which segments are responsible for tissue tropism and virulence. Work with RVFV requires vaccination of at-risk personnel and stringent containment, exceeding BSL-3, to prevent its escape into the environment. This restricts the number of laboratories that are able to pursue studies on RVFV. A model that mimics several of the disease manifestations of Rift Valley fever (RVF), is not severely limited by age-dependency, and does not require extensive containment would facilitate research on this genus.

The prevalence rate in humans for PTV antibody ranges from 10–35% throughout rural Panama where the virus is endemic [11]. While attempting to make antiserum to each of several Punta Toro isolates, James LeDuc noted that adult hamsters died from an acute disease after inoculation with some, but not all isolates (pers. comm.). Previously, Tesh et al. [16], reported blind passage in suckling mice or hamsters as a requirement to establish pathogenicity of strains from western Panama.

In this study, we surveyed isolates from eastern and western Panama to determine whether: (a) the pathogenesis of Punta Toro infection in hamsters mimics the disease manifestations seen with RVF, (b) strains from geographically different regions differ in virulence, and (c) if outcome of infection is virus-host gene dependent.

Materials and methods

Experimental design

Initially, hamsters from the outbred strain, Lak : LVG, were inoculated subcutaneously (sc) to determine virulence of PT virus strains from eastern and western Panama. Secondly, a representative strain (Adames) from eastern and (Balliet) from western Panama was inoculated sc (4×10^4 PFU) into two inbred and five outbred hamster strains to examine differences in susceptibility. Thirdly, a sequential study was conducted during the 18 days post inoculation to follow the development of histological lesions, virus tissue titers, and viral antigen expression with the Adames and Balliet PT strains in outbred Lak : LVG hamsters. Hamsters were inoculated sc with either 5×10^5 PFU of the Adames or Balliet strain. A minimum of three hamsters were euthanized at each time point (12, 24, 36, 48, 60 h; 4, 6, 8, 10, 18 d) post inoculation. Tissues were excised and divided with pieces fixed by immersion in 10% neutral buffered formalin for histopathology, embedded in O.C.T. compound (Lab-Tek, Naperville, IL) and frozen for later antigen detection, or frozen at −70 °C for later infectious virus assay. Finally, other species were examined for susceptibility to PT virus infection.

Animals

Seven strains of 10-week-old female hamsters (*Mesocricetus auratus*) were used. Inbred MHA/SsLak, CB/SsLak, LHC/Lak, LSH/SsLak, and PD4/Lak were purchased from Charles River Lakeview, Wilmington, MA. Outbred hamster strains, Lak : LVG (SYR) and Sch (SYR), were obtained from Charles River Lakeview and from ARS/Sprague-Dawley,

Madison, WI, respectively. One strain, Chi : (CHN), of female Chinese hamsters (*Cricetulus griseus*) was obtained from Chick Line Company, Vineland, NJ, and were used at 4–5 weeks of age. Seven strains of inbred female, 4–5-week-old rats (*Rattus norvegicus*) were used. They were obtained from Microbiological Associates, Walkersville, MD (BN/fMai, LEW/fMai, MAXX, F344, ACI), and The Charles River Breeding Laboratories, Inc., Wilmington, MA (WKY/NCrl). Rodent chow and water were provided ad libitum. The observation period for all studies was 30 days.

Virus

Five strains of Punta Toro virus were obtained from James LeDuc, Gorgas Memorial Laboratory, Panama. The Balliet strain was obtained from the Yale Arbovirus Research Unit, Yale University, New Haven, CN. The passage histories of the strains are shown in Table 1. The viruses were stored in aliquots at −70 °C in Eagle's minimal essential medium (EMEM) supplemented with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum and gentamycin (50 mg/l). Dilutions were prepared in Hanks' balanced salt solution (HBSS), buffered to pH 7.4 with HEPES containing 2% heat-inactivated fetal bovine serum and gentamycin. All viral strains were administered sc to groups of not less than five animals, unless noted in the text.

Plaque-reduction neutralization assay

Serum neutralizing antibody titers were determined essentially as described before, except that the second overlay was added on day 4 and the plaques enumerated on day 5 for PTV [2]. Briefly, fourfold serial dilutions of sera in HBSS with HEPES, 2% heat-inactivated fetal bovine serum, and gentamycin were mixed with an equal volume of diluent containing 50–100 PFU of PTV. After 1 h absorption period, residual infectivity was determined by plaquing under agarose. A second overlay containing 0.1 mg/ml neutral red was applied on day 4 post infection. The 80% neutralization titers are expressed as the highest dilution of serum that caused an 80% reduction in PTV plaques.

Infectious virus assay

Decimal dilutions were assayed by inoculating duplicate 16-mm Vero cell monolayers with 50 µl of sample diluted in HBSS with HEPES, 2% heat-inactivated fetal bovine serum, and gentamycin. Plaques were enumerated as in the plaque-reduction neutralization assay.

Punta Toro antigen detection

Punta Toro viral antigens were detected either by an immunofluorescence assay, as previously described [15], or by immunohistochemical staining using mouse hyperimmune ascites fluid and an abidin-biotin-peroxidase (ABC) system [10] with slight modification [4].

Pathology

Hamsters were euthanized with CO_2 and gross pathological changes were noted. Tissues were fixed in 10% neutral buffered formalin by immersion. Paraffin sections were prepared and stained with hematoxylin and eosin (H & E) for examination by light microscopy.

Results

Survey of PTV strains from eastern and western Panama

The three isolates from eastern Panama were highly virulent ($LD_{50} < 1.0 \log_{10}$ PFU) while three isolates from western Panama, including the prototype strain (Balliet), were of low virulence ($LD_{50} > 5.2 \log_{10}$ PFU) for 10-week-old female Lak : LVG (SYR) outbred hamsters inoculated sc (Table 1). Isolates obtained from different sources from these regions (human infections or sandflies) demonstrated similar virulence characteristics for hamsters.

Survey of susceptibility of hamster strains to lethal PTV infection

The prototype strain, Balliet, from western Panama and the Adames strain from eastern Panama were used to screen 4–5-week-old inbred and outbred hamster strains for susceptibility (Table 2). The Balliet strain was not virulent for the two outbred and four inbred strains when inoculated sc. However, only 3/5 CB/SsLak survived an infection of 4.6 $\log_{10}$ PFU, sc. Those that died, had a prolonged survival time compared to the same hamster strain infected with the Adames strain. All survivors had detectable serum neutralizing antibody 30 days post challenge. The Adames strain was virulent for all of the outbred and inbred strains. The Adames strain was also 100% lethal for 10-week-old hamsters of all strains with no appreciable change in the mean time to death (MTD) (data not shown). Overt disease was characterized by a rapid acute onset of lethargy, assumption of a hunched position, and ruffled fur. Death usually ensued within hours of onset of clinical signs. The average survival time (AST) of hamsters inoculated with the virulent PTV strains was approximately the same regardless of the hamster strain tested.

A sequential comparison of viral tissue titers was conducted with 10-week-old Lak : LVG (SYR) hamsters inoculated sc with 5.7 $\log_{10}$ PFU of the Balliet or Adames strains of PTV. The Adames strain produced an acute fatal disease by day 3 post infection with viremia and viral tissue titers of approximately 9

Table 1. Differences in lethality of Punta Toro virus strains for hamsters[a]

Region	Strain	Date isolated	Passage history	Source	$Log_{10}LD_{50}$ (PFU)
Eastern Panama	Adames	1972	$Vero_3$	Human	0.8
	002381	1975	$Vero_3$	Sandfly	0.1
	065990	1976	$Vero_2$	Hamster	<0.1
Western Panama	Balliet	1966	$Sm_{11}Vero_6$	Human	>6.3
	VP-4401	1970	$Vero_3$	Sandfly	>5.2
	VP-321C	1970	$Vero_3$	Sandfly	5.3

[a] Ten-week-old female Lak : LVG outbred hamster obtained from Charles River Lakeview, Wilmington, MA

Table 2. Susceptibility of different hamster strains to Punta Toro virus[a]

Hamster strains	Punta Toro strain			
	Adames		Balliet	
	(sur/inoc)[b]	AST[c]	(sur/inoc)	AST
Outbred				
Lak: LVG (SYR)[d]	0/5	3.8 ± 0.4	5/5	
Sch (SYR)[e]	0/5	3.0 ± 0.0	5/5	
Inbred				
MHA/SsLak[d]	0/5	3.4 ± 0.5	5/5	
LHC/Lak[d]	0/5	3.5 ± 0.6	5/5	
CB/SsLak[d]	1/5	3.3 ± 0.5	2/5	8.5 ± 0.7
LSH/SsLak[d]	0/5	4.0 ± 0.0	5/5	
PD4/Lak[d]	0/5	5.6 ± 1.6	5/5	

[a] 4×10^4 PFU inoculated subcutaneously in 4-5-week-old female hamsters
[b] Survived/inoculated
[c] Average survival time ± standard deviation in days
[d] Obtained from Charles River Lakeview, Wilmington, MA
[e] Obtained from ARS/Sprague-Dawley, Madison, WI

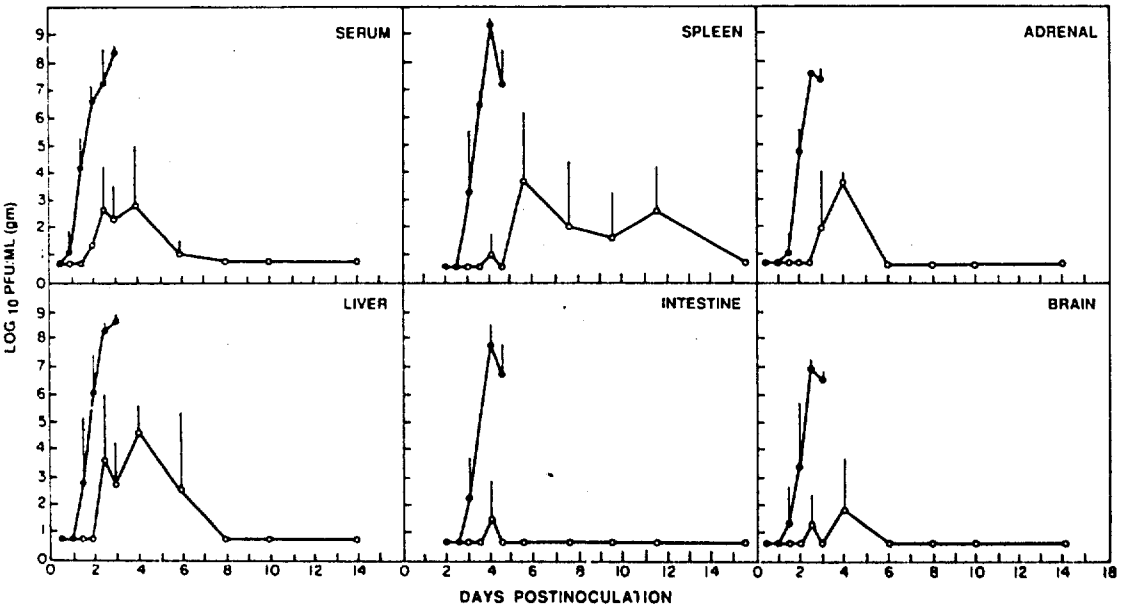

Fig. 1. Comparative viral tissue titers of 10-week-old Lak : LVG (SYR) hamsters inoculated subcutaneously with 5.7 $\log_{10}$ PFU of the Balliet (○) or Adames (●) strain of Punta Toro virus. Each point is a geometric mean ± standard deviation based on the results from three hamsters

$\log_{10}$ PFU/g (ml) of tissue (Fig. 1). In contrast, peak viral titers with the Balliet strain were reduced not only by approximately five orders of magnitude, but delayed as well.

Pathology

Necropsies were performed on infected hamsters in the sequential study. Gross examination of moribund hamsters infected with the Adames strain revealed hemorrhage of the small intestine. When the duodenum was affected, there was a sharp demarcation at the pyloric valve. Distal portions of the small intestine were involved less frequently and extensively. Few microscopic lesions were seen in any tissue prior to 48 h post infection. Intestinal changes present in moribund animals included severe necrosis of the duodenal lamina propria with subsequent sloughing of villous tips. Enterocytes present within crypts were spared (Fig. 2 a). Vessels in affected areas were congested and platelet aggregates were observed in the blood vessels of the villi. PTV antigen, as detected by fluorescence antibody and immunohistochemistry, appeared to be associated with macrophages and endothelial cells, and was also demonstrated in platelet aggregates. Degenerative changes were seen in blood vessels and were characterized by fibrinoid necrosis, margination of leukocytes, pyknosis of endothelial cells, and an occasional rupture which contributed to the hemorrhage (Fig. 2 b). These lesions were not seen with the hamsters infected with the Balliet strain.

The splenic lesions seen with the Adames strain were predominately in the red pulp and consisted of lysis and degeneration of circulating leukocytes, mainly neutrophils, as well as reticuloendothelial cells. Viral antigen, as detected by immunohistochemistry, was seen in mononuclear cells within the marginal zone. Bone marrow from a selected case with pronounced splenic lesions had normal histology. No histological lesions were noted in animals infected with the Balliet strain.

Moderate to severe hepatocellular necrosis was apparent in animals infected with the Adames strain. Antigen was detected in the cytoplasm of necrotic and non-necrotic hepatocytes. In contrast, infection with the Balliet strain was typified by minimal multifocal coagulative necrosis with intense focal infiltration of neutrophils, which were replaced by lymphocytes by day 6 and were absent by day 18 post infection.

Additionally, hamsters infected with the Adames strain had thrombi containing platelet aggregates in vessels of the pancreatic islets, splenic red pulp, and lung. Similarly to that in the intestine, viral antigen was demonstrated in the platelet aggregates of the pancreas. Viral antigen was also seen in macrophages of the sinusoids of the mesenteric lymph node. Necrosis in the zona reticularis of the adrenal was also noted (Fig. 2 c).

Infection of other laboratory rodents

4–5-week-old male Chinese hamsters (*Cricetulus griseus*) were inoculated with the Adames and Balliet strains of PT, sc. The LD_{50} were $< 1.0 \log_{10}$, MTD = 4.0

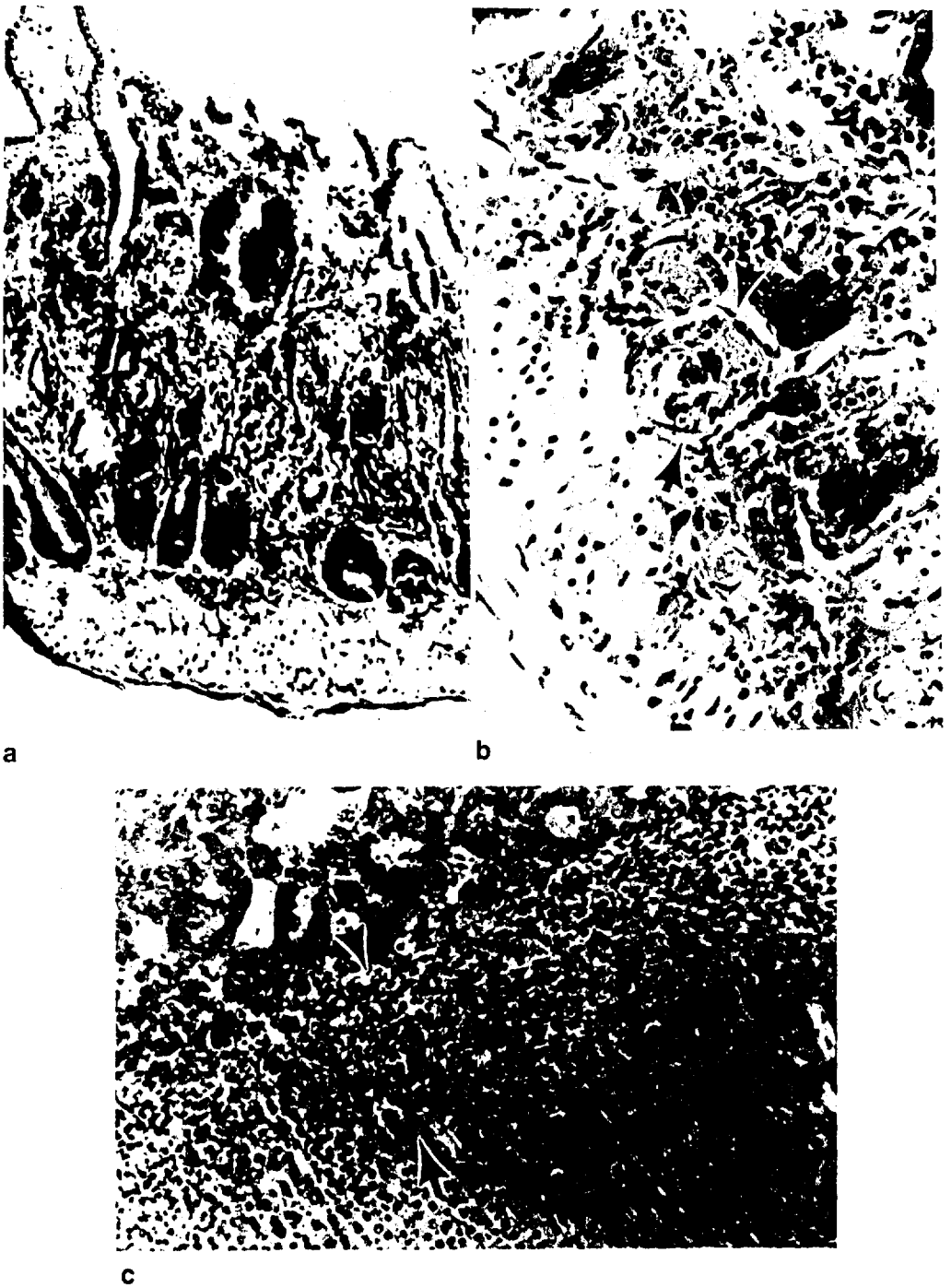

Fig. 2. Ten-week-old Lak : LVG (SYR) hamsters were inoculated with 5.7 $\log_{10}$ PFU of the Adames strain of Punta Toro virus. Tissues were processed for hematoxylin and eosin staining 60 h post infection. **a** Necrosis of the lamina propria and sloughing of villous tip, but not crypt intestinal epithelial cells; **b** vessel wall (between arrows) with early fibrinoid changes, endothelial cells with pyknotic nuclei, and an occasional rupture in the submucosa which contributes to the hemorrhage [H & E]; **c** pyknotic and karyorrhectic nuclei at the zona reticularis (between arrows) in the adrenal [H & E]

days and > 5.7 $\log_{10}$ PFU, respectively. Neither PTV strain was virulent for 4-week-old Tum:(MON) gerbils (*Meriones unguiculatus*) or seven strains (MAXX, Bn/fMai, LEW/fMai, BUF/fMai, F344, ACI, WKY/NCrl) of rats (*Rattus norvegicus*), when inoculated with 4.6 $\log_{10}$ PFU, sc. PT virus was infectious, however, as determinated by detection of serum neutralizing antibodies in the inoculated animals.

Discussion

The PTV-hamster model appears to mimic several of the lesions (liver, spleen, intestinal, and adrenal) found in natural infections with RVFV [7, 9], but differs from the recently published PTV-mouse model [15]. Mice are fully susceptible at 3 weeks but resistant by 7 weeks of age to the Adames strain [15]. The lesions in the mouse and hamster differ by organ and severity. The enteritis was mild in mice and appeared to be confined to the crypt epithelial cells, while severe necrosis of the lamina propria and sloughing of epithelial cells of the villi were seen with hamsters. In the latter, the lesion was associated with platelet thrombi and resulted in ischemic necrosis of the intestine. Splenitis was localized and severe in the red pulp of hamsters, while lesions were more prominent in the white pulp of mice. Necrosis of cells in the adrenal cortex of the hamsters was not reported for mice. Enteritis could be the fatal lesion in hamsters, while hepatocellular necrosis is the salient lesion in mice. In the former, the enteric lesions appear to be of an infarctive nature; viral antigen was seen in platelet aggregates, macrophages of the lamina propria, and possibly endothelial cells. These lesions, and the presence of thrombi in other vessels, strongly suggest that intravascular coagulation, as seen in RVF infections [8, 12], may play a role in PTV infection of the hamster. Viral antigen was abundant in hepatocytes, and macrophages of various organs, suggesting that these two cell types may be the site of most of the viral replication. The hamster provides an adult model where susceptibility can be varied from mild to fatal, depending on the geographical origin of the viral strain.

A survey of three other species (rat, Chinese hamsters, gerbils) demonstrated that the host range appears to be more restrictive for PTV than RVFV, though a fatal infection was induced in Chinese hamsters with the Adames, but not the Balliet strain of PTV. Both strains of PTV infected, but failed to cause overt clinical disease in several inbred rat strains and one outbred gerbil strain, which are highly susceptible to RVFV at 4 weeks of age [3; unpubl. data].

Variation in PTV virulence for hamster may mimic geographical differences seen for RVFV with inbred rat strains [1]. The clear difference in pathogenicity of the PTV strains may allow a determination of those RNA segments responsible for virulence and tissue tropism as reassortants between PTV strains can be produced readily [5]. Therapeutic and prophylactic measures can be investigated without the age-dependent restriction found in the mouse model. The hamster PTV-model complements the PTV-mouse model and should extend

our understanding of phleboviral enteritis, host and viral determinants of resistance, and means of protection.

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences-National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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